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Assignment of Biochemical Functions to Glycosyl Transferase Genes Which Are Essential for Biosynthesis of Exopolysaccharides in *Sphingomonas* Strain S88 and *Rhizobium leguminosarum*

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Glycosyl transferases which recognize identical substrates (nucleotide-sugars and lipid-linked carbohydrates) can substitute for one another in bacterial polysaccharide biosynthesis, even if the enzymes originate in different genera of bacteria. This substitution can be used to identify the substrate specificities of uncharacterized transferase genes. The *spsK* gene of *Sphingomonas* strain S88 and the *pssDE* genes of *Rhizobium leguminosarum* were identified as encoding glucuronosyl-(β1→4)-glucosyl transferases based on reciprocal genetic complementation of mutations in the *spsK* gene and the *pssDE* genes by segments of cloned DNA and by the SpsK-dependent incorporation of radioactive glucose (Glc) and glucuronic acid (GlcA) into lipid-linked disaccharides in EDTA-permeabilized cells. By contrast, glycosyl transferases which form alternative sugar linkages to the same substrate caused inhibition of polysaccharide synthesis or were deleterious or lethal in a foreign host. The negative effects also suggested specific substrate requirements: we propose that *spsL* codes for a glucosyl-(β1→4)-glucuronosyl transferase in *Sphingomonas* and that *pssC* codes for a glucuronosyl-(β1→4)-glucuronosyl transferase in *R. leguminosarum*. Finally, the complementation results indicate the order of attachment of sphingan main-chain sugars to the C_{55}-isoprenylphosphate carrier as -Glc-GlcA-Glc-isoprenylpyrophosphate.

Many species of bacteria synthesize and secrete acidic polysaccharides if supplied with a readily convertible carbon source such as glucose (Glc) and with adequate oxygen. *Xanthomonas campestris*, a plant pathogen, and *Rhizobium leguminosarum*, a nitrogen-fixing, nodule-forming plant symbiont, secrete acidic exopolysaccharides (EPS) which promote plant-microbe interactions. Not only does *X. campestris* secrete xanthan gum in planta, but the polysaccharide is also produced commercially from glucose by large-scale industrial fermentation. Xanthan gum is valuable for controlling the viscosity of aqueous solutions in diverse food and industrial applications. Several members of the bacterial genus *Sphingomonas* (26) also secrete capsular acidic polysaccharides with commercially important rheological properties: gellan, welan, rhamsan, S-88, S-198, S-7, and NW11 (22). To recognize that they share a common core structure, we refer collectively to these polysaccharides as sphingans, after the genus. The carbohydrate structures of the repeat units of xanthan gum, the rhizobial EPS, and the sphingans S-88, S-198, and NW11 are diagramed in Fig. 1.

The assembly of the repeat unit for each of these five polymers begins by transfer of glucose-1-phosphate from UDP-glucose to a C_{55}-isoprenylphosphate (IP) carrier (2, 13, 27). The genes that code for these glucosyl-IP transferases are *spsB* for *Sphingomonas* (27), *gumD* for *X. campestris* (34), and *pssA* for *R. leguminosarum* (35). Based on deduced amino acid sequences, the corresponding gene products are members of a large family of related glycosyl-IP transferases which are very likely to be membrane proteins. Surprisingly, although the N-terminal membrane-interacting domains of these three gene products and the cellular membranes in which they reside appear to be substantially different in composition for each bacterium, the proteins can substitute for one another (1, 27). This substitution or genetic complementation is observed when a foreign gene, which has been cloned into a plasmid and then transferred by conjugal mating into a polysaccharide-deficient mutant bacterium, restores polysaccharide synthesis to the recipient.

Previously, we used genetic complementation and DNA sequencing to characterize a large cluster of 17 genes, including *spsB*, which were isolated from *Sphingomonas* strain S88 and are required for synthesis of capsular polysaccharide (37). Within this cluster we identified three additional gene products (SpsQ, -K, and -L) as putative glycosyl transferases by comparing their deduced protein sequences to those for glycosyl transferase genes from other polysaccharide-secreting bacteria. However, the substrate specificities of SpsQ, -K, and -L could not be determined from the deduced protein sequences, since the sequence similarities to the other transferases were limited. In addition, since the repeat unit of sphingan S-88 has two noncontiguous Glc residues in the main chain, it was not possible to know which Glc was initially added to the carrier IP and, by inference, the order of addition of the remaining sugars. By contrast, the orders of assembly for xanthan gum and the rhizobial EPS are already known from structural analysis of lipid-linked carbohydrate intermediates (2, 13).

The repeat units for sphingans S-88, S-198, and NW11 share three sugar linkages, and S-88 and S-198 also have a fourth

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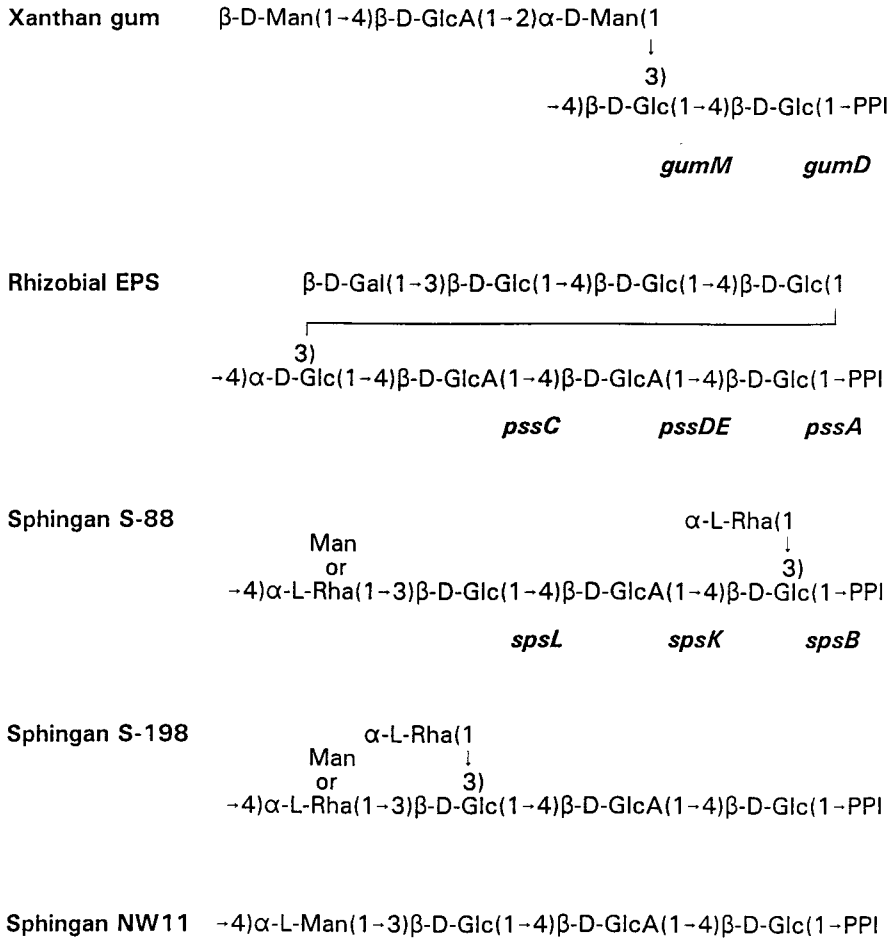

FIG. 1. Biosynthetic genes and structures of repeating units for xanthan gum (14), rhizobial EPS (31), and sphingans S-88 (15), S-198 (4), and NW11 (23). Acetyl, pyruvyl, and hydroxybutanoyl groups are not shown. The reducing end of each repeat is to the right, and the IPP carrier is attached through a phosphodiester linkage at the reducing end. The order of sugars attached to IPP for the sphingans and the corresponding biosynthetic genes were inferred from the genetic and biochemical experiments reported here for strain S88. Gal, galactose; Man, mannose; Rha, rhamnose.

common linkage. Thus, it is reasonable to expect that these closely related strains would have complementary transferases for the common assembly steps. Similarly, by examining the structures of the repeating units of xanthan gum, the rhizobial EPS, and sphingan S-88 (Fig. 1), and assuming the order of assembly for sphingan S-88 as shown, the second glycosyl transferase reactions for *R. leguminosarum* and *Sphingomonas* would be identical and would be different from the second transferase reaction for *X. campestris*. Recently, three putative glycosyl transferase genes from *R. leguminosarum* (*pssC*, *-D*, and *-E*) were isolated and sequenced (35). The deduced amino acid sequence of PssD was found to be similar to that of the amino-terminal half of SpsK, while PssE was similar to the carboxyl half of SpsK. The *pssDE* protein-coding sequences are separated in the genome by only a single base pair (35). In the present work, we analyzed genetic complementation for specific cloned DNA segments isolated from these three genera of bacteria to identify the functions of the individual glycosyl transferase genes of *Sphingomonas* and *R. leguminosarum* and to infer the order of assembly of sphingan S-88. Not only did we observe the expected gene substitutions and restoration of EPS synthesis, but we also discovered that competing foreign glycosyl transferases that form alternative carbohydrate linkages can inhibit either EPS synthesis in the recipient bacterium or bacterial growth.

MATERIALS AND METHODS

Bacterial strains and plasmids. The genotypes of bacterial strains and plasmids used in this study are listed in Table 1.

Culture media. Luria-Bertani medium, YM, and M9 salts are standard media (27), and YMB was described previously (12). Glc (1 to 2% [wt/vol] final concentration) was added to media in agar plates or shake flasks to promote EPS synthesis for *X. campestris* and *Sphingomonas*. The concentrations of antibiotics (Sigma Chemical) used were as follows: rifampin, 20 to 50 μg/ml; streptomycin, 50 μg/ml; and tetracycline, 2 to 15 μg/ml.

Manipulation of cloned glycosyl transferase genes. The glycosyl transferase genes from *Sphingomonas* (*spsQ*, *-K*, *-L*, and *-B*) were cloned from strain S88 by complementation of Sps$^-$ mutants m260, m265, m302, and m54 (37). Smaller segments were prepared from the original cosmid clones pS88c1Δ3, pS88c2, and pS88c3 by standard methods of DNA isolation, digestion with restriction enzymes, and ligation to multiple-cloning sites within the vector DNA (21). Plasmid pB608 was constructed by ligating the *spsK*-containing *Bgl*II segment (nucleotides 7038 through 9165 from the *Sphingomonas* DNA sequence [GenBank accession no. U51197]) obtained from pS88c2 to the *Bgl*II site in pMP92. Plasmid pB554 was assembled by ligating an *Eco*RI *spsB*-containing segment (*Sphingomonas* nucleotides 20249 through 24648) obtained from pS88c3 to pB608. Plasmid pB610 contained a *gumKLM* segment (nucleotides 11643 through 15832 of the *X. campestris* DNA sequence [GenBank accession no. U22511]) which was obtained from plasmid pXCc8 by restriction with *Apa*I and was inserted by blunt-end ligation between the *Bgl*II sites of plasmid pB554 with coincident removal of the resident *spsK Bgl*II segment. The *gumKLM* genes code, respec-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or phenotype	Source (reference)
X. campestris		
X59 (ATCC 55298)	B1459S-4L Rif^r Gum$^+$	These authors (33)
m31	X59 GumD$^-$	These authors (33)
Sphingomonas		
S88	Str^r Sps$^+$	ATCC 31554 (17)
m265 and m260	S88 SpsB$^-$	These authors (27)
m302	S88 SpsB$^-$K$^-$	These authors (37)
R. leguminosarum bv. trifolii		
RBL5515	LPR5 cured of Sym plasmid, Str^r Rif^r	C. A. Wijffelman (28)
RBL5523	RBL5515 pRL1JI *spc-3*::Tn*1831* Pss$^+$	C. A. Wijffelman (25)
RBL5807	RBL5523 Str^r Rif^r *pssA4*::Tn*5* (exo4)	C. A. Wijffelman (35)
RBL5833	RBL5523 Str^r Rif^r *pssD111*::Tn*5* (exo111)	C. A. Wijffelman (35)
E. coli K-12		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 relA1 supE44 hsdR17*(r_K^- m_K^+)Δ(*lacZYA-argF*)*U169*	Bethesda Research Laboratories
HMS174	F$^-$ *hsdR19* (r_K^- m_K^+) *recA1 rpoB331* (Rif^r)IN(*rrnD-rrnE*)*1*	W. Studier
Plasmids		
pRK2013	Ori(ColE1) Kan^r OriT(Mob$^+$) Tra$^+$	D. Helinski (9)
pRK311	OriV(RK2) Tet^r OriT(Mob$^+$) Tra$^-$ λ*cos lacZ*(α)	D. Helinski (6)
pSEB24	OriV(RSF1010) Cam^r Amp^r OriT(Mob$^+$) Tra$^-$	These authors (37)
pMP92	OriV(RK2) Tet^r OriT(Mob$^+$) Tra$^-$	C. A. Wijffelman (32)
pMP2642	*pssA* (RBL5515 DNA in pMP92)	These authors (35)
pMP3030	*pssCDE* (RBL5515 DNA in pMP92)	These authors (35)
pXCc8	*gumBCDEFGHIJKLMN* (X59 DNA in pRK311)	These authors (33)
pXC1483	*gumDEF* (subclone of pXCc8)	These authors (33)
pS88c1Δ3	*spsDCEB rhsACBD* (S88 DNA in pRK311)	These authors (37)
pS88c2	*spsGSRQIKLJFDCEB* (S88 DNA in pRK311)	These authors (37)
pS88c3	*spsQIKLJFDCEB rhsACBD* (S88 DNA in pRK311)	These authors (37)
pZ206	pS88c2-*spsQ*::mini-Tn*10kan*	These authors (37)
pY976	pS88c3-*spsI*::mini-Tn*10kan*	These authors (37)
pY882	pS88c3-*spsK*::mini-Tn*10kan*	These authors (37)
pY872	pS88c3-*spsL*::mini-Tn*10kan*	These authors (37)
pB215	*spsB* (subclone of pS88c3 in pSEB24)	These authors (37)
pB608	*spsK* (S88 DNA in pMP92)	This work
pB554	*spsKB* (S88 DNA in pMP92)	This work
pB610	*gumKLM* SpsB (X59 and S88 DNA in pMP92)	This work
pB611	*gumKLM* (X59 DNA in pMP92)	This work
pB597	*pssCDE spsB* (S88 DNA and pMP3030 subclone in pMP92	This work
pB599	*pssDE spsB* (S88 DNA and pMP3030 subclone in pMP92)	This work
pB609	*pssCD spsB* (S88 DNA and pMP3030 subclone in pMP92)	This work

tively, for glucuronosyl-(α1→2)-mannosyl transferase, mannosyl pyruvylase, and glucosyl-(β1→4)-glucosyl transferase (34). Plasmid pB611 was constructed by deleting the *spsB*-containing *Eco*RI segment from pB610. Plasmid pB597 (*pssCDE spsB*) was obtained by inserting the *spsB*-containing *Eco*RI segment (*Sphingomonas* nucleotides 20249 through 24648) into the *Eco*RI site of pMP3030. Adjacent to *spsB* in this segment is an intact *rhsA* gene which codes for UDP-glucose pyrophosphorylase. Plasmid pB599 (*pssDE spsB*) was obtained by deleting nearly the entire *pssC* gene between the *Sph*I and *Pst*I sites (35) from pB597 (*pssCDE spsB*). Similarly, plasmid pB609 (*pssCD spsB*) was obtained by deleting an essential carboxyl-terminal end of the *pssE* gene between the *BstE*II and *Mlu*I sites (35) from pB597.

Transformation, conjugation, complementation, and transposon mutagenesis. Transfer of plasmid DNA from *Escherichia coli* to either *Sphingomonas*, *X. campestris*, or *R. leguminosarum*, or from *R. leguminosarum* to *E. coli*, was by triparental conjugal mating (7). Purified plasmid DNA was transferred from *Sphingomonas* or *X. campestris* to *E. coli* by transformation (11). Donor cells containing mobilizable broad-host-range tetracycline-resistant (Tet^r) recombinant plasmids were mixed with helper cells containing the mobilizing pRK2013 plasmid and Sps$^-$ recipient *Sphingomonas* cells in a ratio of 5:2:10 and then were spotted onto YM plates lacking Glc and incubated for 6 to 16 h at 30°C. Exconjugants of *Sphingomonas* were isolated by spreading a loopful of the mating mixture onto YM plates containing 1% Glc, 50 μg of streptomycin per ml, and 12 μg of tetracycline per ml. *Sphingomonas* strains are naturally resistant to streptomycin (Str^r), while the donor and helper *E. coli* strains are sensitive. Similarly, exconjugants of *X. campestris* were isolated on YM plates containing 1% glucose, 50 μg of rifampin per ml, and 12 μg of tetracycline per ml, and exconjugants of *R. leguminosarum* were isolated on YMB plates containing 20 μg of rifampin per ml and 2 μg of tetracycline per ml. Mutagenesis by transposition in the nonsuppressing host HMS174 was with Tn*10* derivative 103 (mini-Tn*10 kan*/P_{tac}-ATS transposase) carried by λNK1316 (19).

Chemical analysis of EPS. EPS were precipitated from culture medium with 2 to 3 volumes of isopropyl alcohol or ethanol. The precipitates were dried at 80°C and weighed. Hydrolysis mixtures containing 0.5 to 5 mg of polysaccharide and 130 to 260 μl of 2 M trifluoroacetic acid in high-performance liquid chromatography (HPLC) water were incubated for 4 to 16 h at 95°C and then dried under vacuum, resuspended in 100 μl of HPLC water, dried again, and finally resuspended in 100 μl of HPLC water. Samples and sugar standards were separated on a CarboPac PA-1 anion-exchange column, and compositions were quantitated with a Dionex DX500 HPLC system as described previously (5).

Labeling and chromatographic separation of lipid-linked saccharides. EDTA-treated *E. coli* cells carrying either plasmid B608 (SpsK$^+$) or the vector (pMP92) alone were prepared as follows. The cultures were shaken at 37°C in Luria-Bertani medium to an absorbance (at 600 nm) of about 1.8 and then were chilled on ice and washed twice by centrifugation and resuspension with 1 volume of cold 0.9% NaCl. The cell pellet was resuspended in 10 mM EDTA-Tris (10) at 1/20 of the original culture volume and frozen at −80°C. A sample was thawed at 16°C, centrifuged in the cold, resuspended in 50 mM Tris (pH 8.2)–5 mM EDTA, and then frozen and thawed two more times. The protein concentrations for the EDTA-treated cells were determined with the Bio-Rad protein assay. A standard radioactive labeling reaction mixture contained 0.3 ml of EDTA-

treated cells, 0.3 ml of 50 mM Tris-HCl (pH 8.2)–20 mM $MgCl_2$, and 15 μl (943 pmol) of [^{14}C]UDP-Glc or [^{14}C]UDP-glucuronic acid ([^{14}C]UDP-GlcA) (300 nCi; 318 mCi/mmol), and the reaction mixture was incubated for 60 min at 16°C. Unlabeled UDP-Glc (300 pmol) was added with the labeled UDP-GlcA. After labeling, the permeabilized cells were centrifuged at 14,000 rpm for 2 min in a Hermle 2360K refrigerated microcentrifuge and then resuspended in 1 ml of cold 10 mM EDTA-Tris, and this washing step was repeated two more times. Lipid-linked saccharides were extracted from the cell pellets by adding 0.1 ml of chloroform-methanol-H_2O (1:2:0.1), pooling three extractions, and drying (2). Saccharides were released from the lipids by cleaving the sugar-phosphate bonds with mild acid (50 μl of 0.01 M HCl at 100°C for 10 min), reneutralized, and then separated by gel filtration through a Bio-Gel P column (100 by 1 cm) with 0.1 M pyridinium acetate buffer (pH 5.0) at a rate of about 0.05 ml/min and 0.5 ml/fraction (2). Markers included blue dextran (exclusion), $CoCl_2$ (inclusion), and maltotetraose, maltotriose, maltose, Glc, and glucuronic acid (GlcA). Unlabeled sugars were detected by the phenol-sulfuric acid method (8).

RESULTS AND DISCUSSION

Related *Sphingomonas* strains have gene products analogous to the SpsB, -K, and -L gene products of strain S88. DNA sequencing of a cluster of genes required for sphingan synthesis by strain S88 and comparison of the deduced protein sequences to those of gene products involved in polysaccharide synthesis in other bacteria revealed four genes (*spsQ*, *-K*, *-L*, and *-B*) whose products were likely glycosyl transferases (37). Our strategy for determining the substrate recognition properties of these gene products in vivo was to transfer genes between related strains of *Sphingomonas* which synthesize similar but distinct polysaccharides and to observe whether sphingan synthesis was restored to polysaccharide-negative recipients. Random segments of the NW11 chromosome were inserted into a broad-host-range cosmid vector and screened for clones which restored sphingan synthesis to an SpsB$^-$ mutant of strain S88. One such clone (pNWc1) also restored sphingan synthesis to *spsK* and *-L* mutants of strain S88. However, mutations in *spsQ* in strain S88 were not complemented by pNWc1, despite the presence of sufficient coding potential on the cloned segment to the right and left of the *spsKLB* region. By contrast, the *spsQ*, *-K*, *-L*, and *-B* mutations in S88 were complemented by pS198c5, which carries a chromosomal segment cloned from strain S198. In addition DNA hybridization tests (data not shown) indicated that the clone from strain S198 had segments that hybridized to gene-specific probes for the *spsB*, *-K*, and *-L* genes of strain S88. These initial complementation and hybridization results suggested that the SpsB, -K, and -L gene products in strain S88 and the analogous enzymes in NW11 and S198 were conserved and probably catalyzed biosynthetic steps which were common to these strains. Since we knew that *spsB* coded for a glucosyl-IP transferase (27), the *spsK* and *-L* genes were likely to code for the other two common glycosyl transferases, the glucuronosyl-(β1→4)-glucosyl and glucosyl-(β1→4)-glucuronosyl transferases. However, which one codes for each transferase is not revealed by these complementation tests alone. By elimination, we propose that the SpsQ function, which also has a transferase-like protein sequence (37), is probably involved in adding L-rhamnose or L-mannose to the common trisaccharide core in strains S88 and S198 and is replaced by a different gene product in NW11.

Reciprocal complementation between the *Sphingomonas spsK* and *R. leguminosarum pssDE* genes. A comparison of deduced amino acid sequences for the SpsK and PssDE products shows significant partial homology, suggesting a common function (35). Since a stable lipid-linked trisaccharide with the structure GlcA-GlcA-Glc-isoprenylpyrophosphate was isolated from *R. leguminosarum*, the first transferase attaches glucose-1-phosphate to the carrier IP and the second transferase attaches GlcA to Glc-PPI (2). These two reactions may be identical to assembly steps in *Sphingomonas*, depending on which Glc of the sphingans is added first to the carrier. However, as shown in Fig. 1, assembly of the sphingans and the rhizobial EPS must diverge with the third transferase reaction, where different substrates are recognized. Therefore, we tested whether PssDE and SpsK could complement one another.

The relative frequencies for conjugal transfer of specific plasmids carrying the *spsK* and *pssDE* genes into three recipients (*Sphingomonas* strain S88, *X. campestris*, and *R. leguminosarum*) and phenotypes of the exconjugants were determined (Table 2). When *Sphingomonas* strain S88 was the recipient, sphingan was produced by mutant m265 (SpsB$^-$) only when a functional glucosyl-IP transferase was present (plasmids pB215, pB554, and pB599). Similarly, the *pssDE* genes (pB599) substituted for the SpsK$^-$ defect in mutant m302, allowing synthesis of polysaccharide which was not distinguishable by sugar composition from that made by the native *spsK* gene (pB554). Chromatograms of both the sphingan controls and these recombinant-derived products showed characteristic peaks of Glc, mannose, GlcA, and rhamnose (data not shown), as expected for sphingan S-88. In the reciprocal transfer, the *spsK* gene (pB608) at least partially restored synthesis of rhizobial EPS in a PssDE$^-$ mutant (RBL5833), and an intermediate colonial phenotype was observed. The amount of EPS produced was less than the amount secreted by wild-type *R. leguminosarum* (RBL5523). Complementation was eliminated by a mini-Tn*10* insertion in *spsK* (pY882). The sugar compositions of the EPS from strain RBL5833 carrying either the normal *pssCDE* or foreign *spsK* genes appeared to be identical (data not shown) and matched the rhizobial EPS pattern. The most likely explanation is that *spsK* and *pssDE* code for functionally equivalent transferases that add GlcA to Glc-PPI. The results of the following labeling experiments are consistent with this hypothesis.

Glycosyl transferase-specific labeling of lipid-linked saccharides. Incorporation of radioactive Glc and GlcA into lipid-linked disaccharides was used to identify the substrates recognized by the SpsK glycosyl transferase. EDTA-permeabilized cells of *E. coli* DH5α carrying either pB608 with the *spsK* gene or the vector (pMP92) alone were labeled with radioactive [^{14}C]UDP-Glc or [^{14}C]UDP-GlcA. The *spsK* fragment cloned in pB608 included the normal *Sphingomonas* promoter and ribosome-binding region upstream from the *spsK* gene and small incomplete segments of the flanking *spsI* and *spsL* genes. Therefore, the activity of the SpsK protein in *E. coli* depended on gene expression in the foreign host and on the production of the other putative substrate, Glc-PPI. Labeled saccharides were released from lipid-linked intermediates in the cell membranes and separated by gel filtration into mono-, di-, and oligosaccharides.

The chromatographic elution profiles are shown in Fig. 2. The permeabilized bacteria used for Fig. 2A and B were labeled with [^{14}C]UDP-Glc. It was not surprising to find counts in the monosaccharide peak, since *E. coli* has at least two transferases that can attach Glc to lipids: the first transferase in colanic acid biosynthesis (16) and a potent membrane-associated activity involved in the synthesis of membrane-derived oligosaccharides (18). The activity of the SpsK gene product caused a reproducible increase in counts in the disaccharide peak in Fig. 2B. The increase was 90%, and in a replicate labeling the increase was 80%. This is consistent with Glc-PPI, produced endogenously by *E. coli*, as a substrate for the SpsK transferase. Figure 2C and D show that incorporation of radioactive GlcA into lipid-linked saccharides from [^{14}C]UDP-GlcA was much lower than from UDP-Glc, but as before we observed SpsK-specific labeling of disaccharides, consistent

TABLE 2. Expression of glycosyl transferase genes in *Sphingomonas*, *X. campestris*, and *R. leguminosarum*

Recipient strain (phenotype)	Donor plasmid (genotype)[a]	Exconjugants	
		Frequency	Morphology[b]
Sphingomonas S88			
m265 (SpsB$^-$)	pB215 (*spsB*)	Normal	+
	pB554 (*spsKB*)	Normal	+
	pB599 (*pssDE spsB*)	Normal	+
	pB610 (*gumM spsB*)	Normal	−
	pB611 (*gumM*)	Normal	−
	pB597 (*pssCDE spsB*)	Normal	Tiny
	pB609 (*pssC spsB*)	Normal	Tiny
	pMP3030 (*pssCDE*)	Normal	−
m302 (SpsK$^-$B$^-$)	pB554 (*spsKB*)	Normal	+ (S-88)
	pS88c3 (*spsQIKLB*)	Normal	+
	pY976 (*spsQI*::Tn*KLB*)	Normal	+
	pY872 (*spsQIKL*::Tn*B*)	Normal	+
	pY882 (*spsQIK*::Tn*LB*)	Normal	−
	pB599 (*pssDE spsB*)	Normal	+ (S-88)
	pB610 (*gumM spsB*)	Normal	−
	pB611 (*gumM*)	Normal	−
	pB597 (*pssCDE spsB*)	Normal	Tiny
	pB609 (*pssC spsB*)	Normal	Small
	pMP3030 (*pssCDE*)	Normal	−
X. campestris X59 m31 (GumD$^-$)	pSY1483 (*gumDEF*)	Normal	+
	pS88c1Δ3 (*spsB*)	Normal	+
	pS88c2 (*spsQIKLB*)	Zero	ND
	pS88c3 (*spsQIKLB*)	Zero	ND
	pZ206 (*spsQ*::Tn*IKLB*)	Zero	ND
	pY976 (*spsQI*::Tn*KLB*)	Zero	ND
	pY872 (*spsQIKL*::Tn*B*)	Zero	ND
	pY882 (*spsQIK*::Tn*LB*)	Normal	+ (XG)
	pB610 (*gumM spsB*)	Normal	+
	pB608 (*spsK*)	Normal	−
	pB554 (*spsKB*)	Zero	ND
	pB599 (*pssDE spsB*)	Zero	ND
	pB609 (*pssC spsB*)	Normal	+ (XG)
R. leguminosarum			
RBL5833 (PssDE$^-$)	pMP3030 (*pssCDE*)	Normal	+ (rhEPS)
	pMP2642 (*pssA*)	Normal	−
	pB608 (*spsK*)	Normal	Intermediate (rhEPS)
	pS88c3 (*spsQIKLB*)	Low	Intermediate/− (1/9)
	pY872 (*spsQIKL*::Tn*B*)	Normal	Intermediate (rhEPS)
	pY882 (*spsQIK*::Tn*LB*)	Normal	−
	pB610 (*gumM spsB*)	Zero	ND
	pB611 (*gumM*)	Zero	ND
RBL5523 (Pss$^+$)	pMP3030 (*pssCDE*)	Normal	+
	pMP2642 (*pssA*)	Normal	+
	pS88c3 (*spsQIKLB*)	Low	+
	pY872 (*spsQIKL*::Tn*B*)	Normal	+
	pY882 (*spsQIK*::Tn*LB*)	Normal	+, Small
RBL5807 (PssA$^-$)	pMP3030 (*pssCDE*)	Normal	−
	pMP2642 (*pssA*)	Normal	+
	pB608 (*spsK*)	Normal	−
	pB554 (*spsKB*)	Normal	+
	pS88c3 (*spsQIKLB*)	Low	+/− (1/9)
	pY872 (*spsQIKL*::Tn*B*)	Normal	+
	pY882 (*spsQIK*::Tn*LB*)	Normal	+/− (9/1)
	pB610 (*gumM spsB*)	Zero	ND
	pB611 (*gumM*)	Normal	−

[a] Only the relevant genes are listed; see Table 1 for a complete list of genes on plasmids.

[b] EPS-producing colonies of *Sphingomonas*, *X. campestris*, and *R. leguminosarum* are denoted as +, and nonproducers are denoted as −; intermediate for *R. leguminosarum* indicates a colonial appearance between smooth (+) and rough (−). XG, rhEPS, and S-88 indicate the sugar compositions typical of xanthan gum, rhizobial EPS, and sphingan S-88, respectively. Tiny and small refer to reduced colony sizes. The relative frequency of colony types is given in parentheses. Zero means that no exconjugants were observed. ND, not determinable by visual inspection. The position of each mini-Tn*10kan* insertion was determined by digestion with restriction endonucleases and comparison to the DNA sequence of segment S88c3 (37).

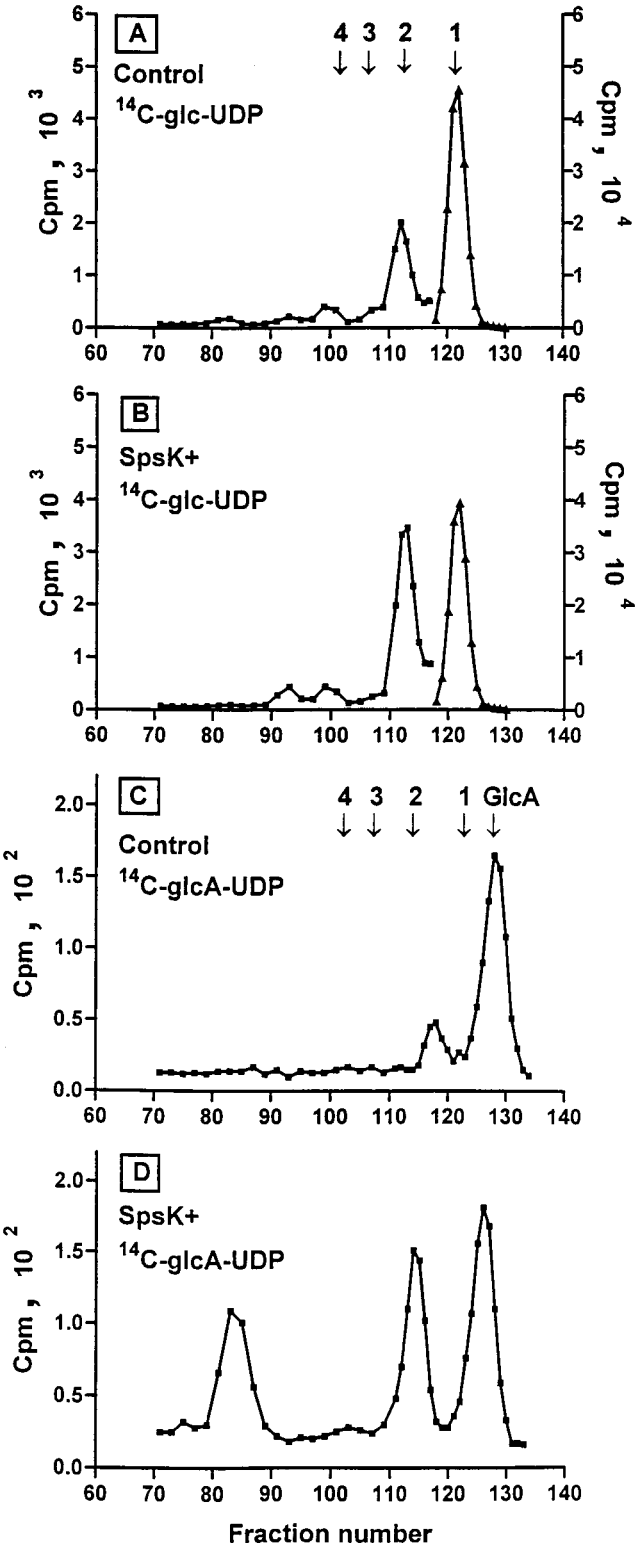

FIG. 2. Gel filtration of saccharides released from lipid-linked intermediates. (A) Control plasmid vector pMP92 labeled with [^{14}C]Glc-UDP; (B) SpsK$^+$ plasmid pB608 labeled with [^{14}C]Glc-UDP; (C) pMP92 labeled with [^{14}C]GlcA-UDP; (D) pB608 labeled with [^{14}C]GlcA-UDP. The scale on the right for panels A and B applies to fractions 117 to 130. The arrows show the positions of marker saccharides: GlcA, Glc (arrow 1), maltose (arrow 2), maltotriose (arrow 3), and maltotetraose (arrow 4).

with UDP-GlcA being a substrate for SpsK. The profile in Fig. 2D also shows a species of higher mobility that is also SpsK specific, but the size and nature of the saccharide are not known. Taken together, the in vivo genetic complementation tests and the in vitro sugar incorporation studies indicate that the repeating subunits for the sphingans are assembled from nucleotide sugars to give the intermediate structure Glc-GlcA-Glc-PPI. The first transferase (SpsB) adds glucose-1-phosphate to the IP carrier, and then SpsK adds GlcA to Glc-PPI. For the rhizobial EPS intermediate structure, GlcA-GlcA-Glc-PPI (2), PssA adds glucose-1-phosphate to IP and PssDE adds GlcA to Glc-PPI.

Genetic transfers resulting in inhibition of polysaccharide synthesis or cellular growth. By contrast with the straightforward positive complementation between two genes which are functionally equivalent, we also observed "negative" phenomena associated with the transfer of certain glycosyl transferase genes, i.e., inhibition of polysaccharide synthesis or cell growth. In earlier work we caused mutated *Sphingomonas* bacteria, which carried an extensive deletion of the *sps* genes, to secrete copious amounts of xanthan gum by transferring to the recipient a cluster of 12 biosynthetic genes (*gumBCDEFGHIJKLM*) from *X. campestris* (36). However, as we show in Table 2, we failed to achieve a reciprocal genetic transfer, and specifically, plasmids carrying *Sphingomonas* genes could not be recovered intact from *X. campestris* if the incoming DNA included a functional *spsK* gene. *X. campestris* mutant m31 was complemented by either a *gumD* gene (pSY1483) or an *spsB* gene (pS88c1Δ3, pB610, pB609, and pY882), and the exconjugants produced xanthan gum of normal composition. Notably, the *spsK* gene in plasmid pY882 included a gene-inactivating mini-Tn*10* insertion. By contrast, plasmids carrying both an *spsB* gene and an *spsK* gene (pS88c2, pS88c3, pZ206, pY976, pY872, and pB554) were not recovered. The detrimental effect of *spsK* in *X. campestris* required the presence of a functional glucosyl-IP transferase such as SpsB, since the *spsK* gene alone (pB608) was not detrimental and remained intact as determined by restriction endonuclease analysis. Likewise, the *pssDE* genes could not be transferred and maintained in *X. campestris* m31 when a functional glucosyl-IP transferase was included (pB599). For the pB599 mating we did observe a single mucoid Gum$^+$ colony after conjugal mating, but upon plasmid isolation and restriction analysis we found that the plasmid had the *pssDE* genes deleted and failed to complement the Sps$^-$ *Sphingomonas* strain m302, unlike intact plasmid pB599. These results indicate that glycosyl transferases like SpsK and PssDE, which create an unnatural lipid-linked saccharide in *X. campestris*, are toxic to these cells.

Transfer into *Sphingomonas* strain m265 (SpsB$^-$) of the *spsB* and *gumM* genes (pB610) did not prevent cell growth but eliminated complementation by *spsB*, resulting in no sphingan synthesis. Similar inhibition of sphingan synthesis was observed when the *gumM* gene on pB610 was transferred into the SpsK$^-$B$^-$ *Sphingomonas* strain m302. The *gumM* gene caused a large reduction in the recovery of exconjugants in *R. leguminosarum*, as long as the recipient carried a glucosyl-IP transferase, such as PssA or SpsB (RBL5833pB611 or RBL5807 pB610, respectively). In the absence of Glc-PPI, the GumM product was not toxic [RBL5807(pB611)]. When plasmids pB610 (SpsB GumKLM) and pB611 (GumKLM) were transferred separately to an *R. leguminosarum* mutant which synthesizes small amounts of rhizobial EPS (data not shown), only plasmid pB610 was toxic. This suggests that the degree of toxicity may depend on the number of incorrectly linked lipid carriers that accumulate.

Plasmids carrying the *pssCDE* (pMP3030) and *spsK* (pB608)

genes were transferred normally to *R. leguminosarum* and were stable. By contrast, plasmid pS88c3, with its additional 20 genes from *Sphingomonas*, was poorly transferred into the rhizobial recipient. The plasmids that were subsequently isolated from the rare pS88c3 exconjugants had severe deletions and were missing restriction sites and *spsL* and other genes. The plasmids with the deletions were readily transferred back into the same rhizobial recipients. This suggests that the *spsL* gene is detrimental to *R. leguminosarum*. However, normal recovery of exconjugants was obtained if either the *spsL* gene (pY872) or the complementing *spsK* gene (pY882) on plasmid pS88c3 included a gene-inactivating transposon. The pY882 plasmid caused the Pss^+ RBL5523 recipient to grow as relatively small colonies, as if the plasmid *spsL* gene was expressed at least partially. Similarly, introduction of the *pssC* and *spsB* genes (pB597 and pB609) into *Sphingomonas* strain m265 or m302 caused the exconjugant colonies to be reduced in size, as if the foreign *pssC* gene was toxic to *Sphingomonas*. The detrimental effect was not observed in the absence of a functional glucosyl-IP transferase (pMP3030). The most straightforward interpretation is that both *spsL* and *pssC* code for third transferases but that their products attach different sugars to GlcA-Glc-PPI.

Deleterious effects of incomplete or unnatural lipid-linked saccharides in bacteria. Although secreted polysaccharides are not usually essential for cell viability, accumulation of lipid-linked intermediates in the cytoplasmic membrane appears to be harmful. For example, in *Salmonella enterica*, absence of the O antigen gives colonies a rough appearance compared to the normal smooth phenotype, and most of the rough mutants that are spontaneous or isolated after chemical mutagenesis are defective either in *wbaP* (formerly *rfbP*; coding for a galactosyl-IP transferase as the first step in assembly of the repeat) or in synthesis of the O-antigen precursor dTDP-rhamnose (20). Interestingly, a mutation (*rfbH819*) that blocks the synthesis of CDP-abequose gives rise to many lipopolysaccharide-positive revertants and secondary *wbaP* mutations (38), and in a similar way, phosphomannoisomerase mutants which do not synthesize GDP-mannose also acquire secondary *rfbP* mutations (20). As a second example, in *Rhizobium meliloti*, mutations in *exoP*, *-T*, *-Q*, *-L*, and *-M* are lethal in cells that are derepressed for succinoglycan synthesis (29). Based on protein sequence similarities, ExoP, -T, and -Q appear to be involved in secretion, while ExoL and -M code for distinct glucosyl-(β1→4)-glucosyl transferases that attach the third and fourth sugars of the succinoglycan repeat (30). However, in contrast to the above-described examples, in *X. campestris* mutations in the glycosyl transferase genes (*gumD*, *-M*, *-H*, *-K*, and *-I*) are not detrimental, while mutations in genes for secretion functions (*gumB*, *-C*, and *-J*) and in the putative polymerase gene (*gumE*) are lethal (3, 34). For *Sphingomonas*, we also find that most recovered Sps^- mutations are in the *spsB* gene, which codes for the glucosyl-IP transferase, and the only mutants we have so far isolated that have mutations in the *spsK*, *spsL*, and *rhs* operon have second mutations in *spsB*, as if the failure to start assembly of the repeat unit restored full viability to these mutants.

Bacteria might have mechanisms to release the IP carrier from incomplete repeat subunits so that it can be used for the synthesis of essential peptidoglycan. However, we find that synthesis of nonnative repeat structures may be especially detrimental: the *spsK* and *pssDE* genes were toxic to *X. campestris*, the *gumM* and *spsL* genes were detrimental to *R. leguminosarum*, the *pssC* gene was toxic to *Sphingomonas*, and the *gumM* gene inhibited sphingan synthesis in *Sphingomonas* and rhizobial EPS synthesis in *R. leguminosarum*. We suggest that the formation of a nonnative oligosaccharide linked to the lipid carrier is toxic because the carrier cannot be released for other essential cellular functions.

Overview. As shown in this work, the substrate requirements of glycosyl transferases involved in EPS biosynthesis can be determined in vivo by cloning and expression of foreign transferase genes in a recipient host, as long as the essential nucleotide-sugar precursors are present. The applicability of this in vivo approach will broaden as the substrate specificities of additional glycosyl transferase genes are determined and the genes and corresponding polysaccharide-negative mutant bacteria become available. An alternative approach is isolation and characterization of radiolabeled lipid-linked intermediates that accumulate in the membrane fractions of permeabilized bacteria. This approach depends on labeled nucleotide sugars, some of which are not readily available, and yields definitive results when only one polysaccharide structure is assembled (16). More importantly, the requirement for an organized membrane apparatus may cause a loss of specificity, and the extracts may accumulate only a few of the incomplete subunit forms (24).

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